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(54) **Protein forming a water channel and gene therefor.**

(57) A WCH-1 cDNA probe specific to mRNA expressing a water channel localized in the kidney collecting tubule and complementary to said mRNA, and the sequence are described. Same is obtainable by

a) subjecting a single-chain cDNA prepared from kidney medullary mRNA of a mammal, preferably a rat, to PCR using, as degenerate primers,

5' - (T/C)T(T/C/A/G)AA(T/C)CC(T/C/A/G)GC(T/C/A/G)GT

(T/C/A/G)AC- 3'

and

5' - AA(T/C/A/G)(G/C)(T/A)(T/C/A/G)C(G/T)(T/C/A/G)GC(T/C/A/G)

GG(A/G)TT- 3',

and

b) screening a kidney cDNA library of said mammal using a product of said PCR as a probe.

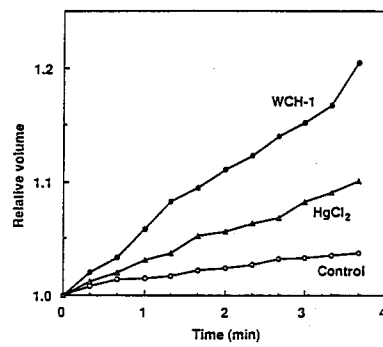
WCH-1 protein molecules constituting said water channel can be produced by Escherichia coli producing protein molecules expressed by the WCH-1 gene.

The figure shows an increase in osmotic water permeability of Xenopus oocytes in which WCH-1 RNA has

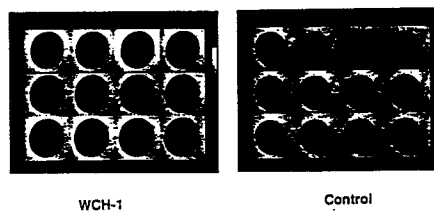
been injected compared to a control sample.

Fig. 8

(a)



(b)



This invention relates to a gene and protein molecules forming a vasopressin-regulated water channel WCH-1 localized in the kidney collecting tubule.

Urine concentration is mandatory for most mammals in order to prevent loss of body water. Concentrated urine is produced, in response to vasopressin, by the transepithelial water recovery from the lumen of the kidney collecting tubule through high water permeable membranes (Orloff, J. & Handler, J. S. Am. J. Med. 42, 757 - 768 (1967); Knepper, M. A. & Rector, F. C. Jr. in The Kidney (eds Brenner, B. M. & Rector, F. C. Jr.) 445 - 482 (W. B. Saunders, Philadelphia, (1991))). In this nephron segment, vasopressin regulates water permeability by endo- and exocytosis of water channels from and to the apical membrane (Handler, J. S. Am. J. Physiol. 255, F375 - F382 (1988); Harris, H. W. Jr., Strange, K. & Zeidel, M. L. J. Clin. Invest. 88, 1 - 8 (1991)).

Recently, it has been shown that CHIP 28 is a water channel in red blood cell membranes (RBC) and in kidney proximal tubule (Preston, G. M., Carrol, T. P., Guggino, W. B. & Agre, P. Science 256, 385 - 387 (1992)). However, CHIP 28 is not expressed in the collecting tubule (Denker, B. M., Smith, B. L., Kuhajda, F. P. & Agre, P. J. Biol. Chem. 263, 15634 - 15642 (1988)).

The presence of the water channel in the kidney collecting tubule has not been proved to date. If the presence of the water channel is demonstrated, isolated and identified, the basis for clarification of the basic principle of the kidney may be achieved, and the basis for therapy of a variety of kidney diseases may be found.

It is therefore an object of the present invention to obtain a water channel of the kidney collecting tubule in an isolated form and to obtain its clone or reproduction and means for cloning or producing the same.

This object is solved by the genes, proteins and methods of the independent claims. Further advantageous features, aspects and details of the invention are evident from the dependent claims, the description, the examples and the drawings. The claims are to be understood as a first non-limiting approach to define the invention in general terms.

According to an aspect of the present invention an artificial membrane is provided in which the water channel of the present invention is incorporated.

Using the gene and the protein molecules, a screening method for water diuretics may be established, while a high water-permeable artificial membrane and liposome into which the WCH-1 protein has been incorporated may be produced.

In the present invention, WCH-1 means a vasopressin-regulated water channel localized in the kidney collecting tubule, while the gene capable of expressing such water channel is termed WCH-1 gene.

According to the present invention, the above object may be achieved by isolation of a WCH-1 cDNA probe, Escherichia coli producing WCH-1 protein molecules, WCH-1 protein molecules produced by the Escherichia coli, and a method for producing the WCH-1 protein molecules. Besides, the lipid membrane in which the WCH-1 protein is incorporated is produced.

Concretely the present invention may be summarized as follows:

A WCH-1 cDNA probe which is specific to mRNA expressing the water channel localized in the kidney collecting tubule and which is complementary to said mRNA.

A WCH-1 cDNA probe which is specific to mRNA expressing the vasopressin-regulated water channel and which is complementary to said mRNA.

The clone of the WCH-1 cDNA is obtainable by

a) subjecting a single-chain cDNA prepared from kidney medullary mRNA of a mammal to PCR using, as degenerate primers,

5' - (T/C)T(T/C/A/G)AA(T/C)CC(T/C/A/G)GC(T/C/A/G)GT

(T/C/A/G)AC - 3'

and

5' - AA(T/C/A/G)(G/C)(T/A)(T/C/A/G)C(G/T)(T/C/A/G)GC(T/C/A/G)

GG(A/G)TT - 3',

and

b) screening a kidney cDNA library of said mammal using a product of said PCR as a probe. The kidney is preferably furnished by a rat.

A base sequence of WCH-1 cDNA represented by sequence number 1 (see Appendix: Table of Sequence Description).

A WCH-1 mRNA probe obtainable by employing the WCH-1 cDNA probe as a template.

A WCH-1 protein molecule constituting a water channel localized in the kidney collecting tubule, or a WCH-1 protein molecule constituting a vasopressin-regulated water channel.

An amino acid sequence representing the above-mentioned WCH-1 protein molecule coded by the base sequence shown by sequence number 1.

A recombinant plasmid in which a WCH-1 gene represented by the base sequence shown by the sequence number 1 is incorporated in an expressing vector.

A recombinant plasmid in which said expressing vector is preferably pSPORT and said WCH-1 gene is inserted into sectioned sites of said pSPORT with Not-I and Sal-I.

Escherichia coli producing WCH-1 protein molecules, expressed by the WCH-1 gene, constituting a water channel localized in the kidney collecting tubule, or Escherichia coli producing WCH-1 protein molecule, expressed by the WCH-1 gene, constituting a vasopressin-regulated water channel (The Escherichia coli have been deposited at the Microorganism Laboratory of the Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan, under designation for identification of "Escherichia Coil rWCH-1" under deposition number of FERM P-13171).

The WCH-1 protein molecule produced by said Escherichia coli.

A method for producing the WCH-1 protein molecule wherein the WCH-1 protein molecule is obtainable using said Escherichia coli.

Preferably, said Escherichia coli is :

one obtained by introducing the recombinant plasmid which has been obtained by inserting the WCH-1 gene into the expressing vector pSPORT into Escherichia coli DH10 α for transformation, and more preferably

said Escherichia coli being one containing the plasmid in which said WCH-1 gene is introduced into sectioned sites of pSPORT by Not-I and Sal-I, or

said Escherichia coli being one having the plasmid produced employing the vector (pSPORT), the expression of which may be derived by addition of isopropyl β -D-thiogalactoside (IPTG), host (DH10 α) family.

A lipid membrane containing the WCH-1 protein molecule.

Liposome formed of a lipid membrane containing the WCH-1 protein molecules.

The following meritorious effects may be expected from the present invention.

1) The presence of the water channel in the kidney collecting tubule is clearly demonstrated, isolated and identified, the basis for clarification of the basic principle of the kidney has now been established.

2) Since the basic principle of the kidney is clarified, a new guideline is provided in giving a diagnosis of kidney lesions.

3) Also, since the basic principle of the kidney is clarified, a new guideline is provided in therapy for kidney lesions or a material for therapy of kidney lesions.

4) The WCH-1 water channel may be expressed on a living membrane by introducing WCH-1 cDNA or mRNA into cells.

5) High water-permeable WCH-1 protein may be acquired easily in large quantities by the gene-operated Escherichia coli according to the present invention.

6) A screening method for water diuretics may be established.

7) A high water-permeable artificial membrane in which is incorporated the WCH-1 protein may be produced.

8) High water-permeable artificial liposome in which is incorporated the WCH-1 protein may be produced.

The following description is made by reference to the cloning of cDNA for WCH-1 which is a new water channel of the apical membrane of the kidney collecting tubule. WCH-1 is identical up to 42 % in amino acid sequence to CHIP 28. WCH-1 transcripts are detected only in the kidney collecting tubule. Immunohistochemically, WCH-1 is localized to the apical region of the kidney collecting tubule cells. Expression of WCH-1 in Xenopus oocytes markedly increased osmotic water permeability. Interestingly, dehydration markedly stimulates WCH-1 mRNA in rat kidney, without stimulating CHIP 28 mRNA. The functional expression by WCH-1 and the limited localization of WCH-1 to the apical region of the kidney

collecting tubule suggest that WCH-1 is the vasopressin-regulated water channel.

Fig. 1 is a chart illustrating a hydropathy profile of a presumed amino acid sequence of WCH-1.

Fig. 2(a) is a photo showing results Northern blot analysis of WCH-1 in a variety of rats' tissues. Fig. 2-
 5 (b) is a photo showing results of the Northern blot analysis of WCH-1 in sliced segments of the cortex and medulla of the rat's kidney.

Fig. 3 is a photo showing agarose gel electrophoresis of an RT-PCR product for WCH-1 mRNA for an isolated nephron segment.

The expressions and abbreviations in English are as follows:

10 PCT ; bent part of a proximal tubule,
 TDL ; thin descending leg of the loop of Henle,
 TAL ; thin ascending leg of a medulla,
 MAL ; thick ascending leg of a medulla,
 CCD ; cortex collective duct,
 OMCD ; outer medulla collective duct,
 15 IMCD ; inner medulla collective duct,
 RT(-) ; reaction without reverse transcriptase,

Fig. 4 is a microscopic photo, with a magnification factor of 100, showing the chromosomal tissue of a rat's kidney medulla portion by the fluorescent antibody technique employing anti-WCH1/C,

20 Fig. 5 is a microscopic photo, with a magnification factor of 100, showing a chromosomal tissue by the fluorescent antibody technique employing anti-WCH1/C previously pre-incubated with a corresponding peptide antigen.

Fig. 6 is a microscopic photo, with a magnification factor of 400, showing a chromosomal tissue of a rat's kidney medulla by the fluorescent antibody technique employing anti-WCH1/C.

25 Fig. 7 is a microscopic photo, with a magnification factor of 100, showing a chromosomal tissue of a rat's kidney cortex portion by the fluorescent antibody technique employing anti-WCH1/C.

Fig. 8(a) is a graph showing a time-dependent volumetric increase of oocytes injected with 20 ng of WCH-1 RNA(WCH-1) and with water (for comparison or control).

Fig. 8(b) is a microscopic photo of oocytes injected with WCH-1 RNA or with water (control).

30 Fig. 9 is a photo showing results of the Northern blot analysis showing changes in the amount of WCH-1 and CHIP-28 mRNA in the rat's kidney following prolonged dehydration.

Fig. 10 is a sequence diagram showing the coincidence of the WCH-1 cDNA nucleotide sequence and its presumed amino acid sequence with human CHIP 28.

The abbreviations of the amino acid sequences are as follows:

35 A ; alanine (Ala),
 C ; cysteine (Cys),
 D ; aspartic acid (Asp),
 E ; glutamic acid (Glu),
 F ; phenylalanine (Phe),
 G ; glycine (Gly),
 40 H ; histidine (His),
 I ; isoleucine (Ile),
 K ; lysine (Lys),
 L ; leucine (Leu),
 M ; methionine (Met),
 45 N ; asparagine (Asn),
 P ; proline (pro),
 Q ; glutamine (Glu),
 R ; arginine (Arg),
 S ; serine (Ser),
 50 T ; threonine (Thr),
 V ; valine (Val),
 W ; tryptophane (Trp),
 Y ; tyrosine (Tyr),

55 Isolation and Determination of Base Sequence of WCH-1 mRNA

Rat kidney medulla poly(A)⁺RNA was submitted to reverse transcription and 30 cycles of PCR with 5 μ M each of two degenerate primers,

5' - (T/C)T(T/C/A/G)AA(T/C)CC(T/C/A/
 5 G)GC(T/C/A/G)GT(T/C/A/G)AC- 3'

and

10 5' - AA(T/C/A/G)(G/C)(T/A)(T/C/A/G)C(G/T)(T/C/A/G)GC(T/C/A/G)
 GG(A/G)TT- 3',

15 synthesized based on conserved amino acid sequences of the MIP family (Leu-Asn-Pro-Ala-Val-Thr, Asn-Pro-Ala-Arg-Ser-Phe, respectively) (Wistow, G. J. Pisano, M. M. & Chepelinsky, A. B. Trends Biochem. Sci. 16, 170 - 171 (1991)). The cycle comprised a denaturation step at 94 °C for 1 minute, annealing at 50 °C for one minute and extension at 72 °C for three minutes, followed by a final extension for 7 minutes.

A band of ~ 370 bp PCR products was isolated by gel electrophoresis and subcloned into the plasmid vector PCR 1000 (Invitrogen). 24 clones were sequenced using the fluorescence DNA sequencer 373A (Applied Biosystems) with -21M13 and M13R fluorescent primers.

A clone (pMWC41) was obtained with a 369 bp insert of 58 % nucleotide sequence identity and 45 % deduced amino acid sequence identity to human CHIP 28.

Another clone (prCHIP 28) was a 369 bp insert of 88 % nucleotide sequence identity and 95 % deduced amino acid sequence identity to human CHIP 28.

The clone pMWC41 was used to screen a 2×10^7 recombinant rat kidney cDNA library constructed in the Not-I/Sal-I site of the λ gt 22 vector (BRL). Under stringent condition (hybridization at $6 \times$ SSPE, 50 % formamide, 42 °C; washing at $2 \times$ SSC, 0.5 % SDS, 42 °C), a positive clone (WCH-1) was isolated with ~ 1.4 kb insert.

30 The cDNA insert was subcloned into the Not-I/Sal-I site of the pSPORT vector (BRL) and the base sequence as well as the amino acid sequence was determined by the Sanger dideoxynucleotide chain termination method using Sequenase (USB).

Fig. 10 shows the sequence for WCH-1. Fig. 10 shows nucleotide sequence for the WCH-1 cDNA and alignment of its deduced amino acid sequence with that of human CHIP 28 (Preston, G. M. & Agre, P. Proc. Natl. Acad. Sci. USA. 88, 11110 - 11114 (1991)). Conserved residues are shown in boxes and deduced transmembrane domains (Kyte, J. & Doolittle, R. F. J. Mol. Biol. 157, 105 - 132 (1982)) are underlined.

* indicates consensus sequences for potential N-linked glycosylation sites (Kornfeld, R. & Kornfeld, S. Ann. Rev. Biochem. 54, 631 - 664 (1985)), ∇ , $\diamond$ and $\bullet$ indicate phosphorylation sites for cAMP-dependent protein kinase, for protein kinase C and for casein kinase II, respectively. The poly(A)⁺ track at the end of the cDNA begins at the 14th base after the AATAAA cleavage and polyadenylation sequence.

The first ATG was determined as an initiation codon on the basis of the Kozak's consensus A at position -3 (Kozak, M. Nucl. Acid Res. 15, 8127 - 8146 (1987)) and the sequence identity of the first seven amino acids to human MIP (Pisano, M. M. & Chepelinsky, A. B. Genomics 11, 981 - 990 (1991)).

45 The longest open reading frame encodes a 271-amino acid protein (Mr 28928) with 42.7 % sequence identity with human CHIP 28 and 59.1 % sequence identity with rat MIP (Kent N. A. Shiels, A. Nucl. Acid. Res. 18, 4256 (1990)). Conserved residues in WCH-1 and the members of the MIP family (Pao, G. M. et al., Mol. Microbiol. 5, 33 - 37 (1991)) and internal tandem repeats (Wistow, G. J., Pisano, M. M. & Chepelinsky, A. B. Trends Biochem. Sci. 16, 170 - 171 (1991)) in the WCH-1 sequence suggest that the WCH-1 is a new member of the MIP family.

50 Fig. 1 shows the hydropathy profile of the deduced amino-acid sequence of the WCH-1. The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (Kyte, J. & Doolittle, R. F. J. Mol. Biol. 157, 105 - 132 (1982)) with a window of 12 residues. Hydropathy analysis of the translated protein indicated the presence of the six transmembrane domains similar to CHIP 28. Deduced membrane-spanning domains are numbered from I to VI.

55 Although the experiments have been conducted in the present invention on rats, it is apparent that the WCH-1 cDNA of any other animal species may be obtained by the above-described operations, if such animal species are mammals. It may be premeditated that substantial portions of the base sequence and the amino acid sequence exhibit identity with these other animal species. (It is noted that for instance,

human CHIP 28 and rat CHIP 28 exhibited 88 % base sequence identity.)

Investigation of the Presence of WCH-1 in Tissues

For Northern blot analysis, RNA extracted from several tissues was enriched for poly(A)⁺ tracts and 10 µg per lane was electrophoresed on agarose gels containing formaldehyde. Equal loading and absence of degradation was checked by staining with ethidium bromide. After transfer to nylon membranes, blots were hybridized under high stringency with the WCH-1 cDNA labeled with ³²P, and autoradiographed for 24 to 120 hours.

RT-PCR of dissected nephron segments was performed as described in Moriyama, T., Murphy, H. R., Murtin, B. M. & Garcia-Perez, A. Am. J. Physiol, 258, F1470 - F1474 (1990) and Terada, Y. et al. Am. J. Physiol. 261, F1080 - F1087 (1991). Briefly, 2 mm of dissected nephron segments were submitted to reverse transcription (RT) with random primer. Synthesized cDNA was used for 40 cycles of PCR reaction (94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 3 minutes), with specific 18nt primers for WCH-1 (5'-TGGGATCTATTTACCGG- 3', bases 522 - 539, and 5'- ACAGGCACTCGGGATCAC- 3', bases 1216 - 1233).

PCR products were electrophoresed in 2 % agarose, stained with ethidium bromide, and photographed. For Southern blot analysis, DNA was denatured and transferred to nylon membranes and hybridized with WCH-1 cDNA labeled with ³²P.

Fig. 2 shows photographs of localization of WCH-1.

Fig. 2 a shows Northern blot analysis of WCH-1 expression in different rat tissues, showing WCH-1 transcripts detected only in the lane containing rat kidney mRNA. WCH-1 transcripts were also not detected in lanes other than kidney mRNA by longer autoradiographic exposure up to 120 hours (data not shown).

Fig. 2 b shows Northern blot analysis of WCH-1 expression in sliced sections of rat kidney cortex and medulla. In addition to a major transcript of ~ 1.5 Kb, larger transcripts of 2.8 Kb and 4.4 Kb were detected. These may represent alternative splicing or polyadenylation variants.

The above northern blot analysis revealed that WCH-1 is expressed exclusively in kidney, predominantly in kidney medulla and less in kidney cortex.

Fig. 3 shows agarose gel electrophoresis of RT-PCR products for WCH-1 mRNA on dissected nephron segments. 712 bp bands for WCH-1 were detected in lanes on CCD, OMCD, IMCD.

By Southern blot analysis, specific binding of probes to PCR bands confirmed the identity of the products (data not shown).

Abbreviations are: PCT, proximal convoluted tubule; TDL, thin descending limb of Henle's loop; TAL, thin ascending limb of Henle's loop; MAL, medullary thick ascending limb; CCD, cortical collecting tubule; OMCD, outer medullary collecting tubule; IMCD, inner medullary collecting tubule; RT (-), reaction without reverse transcriptase.

PCR products of 712 bp specific for WCH-1 was detected only in the cortical, and the outer and inner medullary collecting tubule segments, suggesting the limited expression of WCH-1 mRNA in the collecting tubule.

Then, immunohistochemical localization of WCH-1 in a rat kidney was checked. A peptide corresponding to 15 amino acids at the COOH-terminus of WCH-1 (Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Ser-Lys-Ala), with the NH₂terminus of tyrosine, was synthesized, and conjugated with bovine thyroglobulin in accordance with Skowsky, W. R. & Fischer, D. A. J. Lab. Clin. Med. 80, 134 - 144 (1972). The resulting product is termed a conjugate. Using 0.5 ml of the conjugate obtained by mixing the complete Freund's adjuvant into the conjugate (0.2 mg as peptide), a New Zealand white rabbit was immunized to obtain a rabbit anti-serum (termed anti-WCH1/C hereinafter).

4 µm sections of a fixed kidney of a rat restricted in water intake for two days were mounted on slides, preincubated with a non-immune goat serum, and rinsed. The slides were incubated overnight at 4 °C with 500 : 1 diluted anti-WCH1/C and rinsed. The rinsed slides were incubated with a 1 : 100 diluted solution of FITC-conjugated goat-anti-rabbit immunoglobulin at 25 °C for one hour and stained. The stained slides were rinsed and photographed (Figs. 4 to 7). Immunostaining with anti-sera from three of five immunized rabbits (anti-WCH1/C) produced similar results.

Fig. 4 shows the rat kidney medulla portion incubated with anti-WCH1/C by the above procedure with a magnification factor of 100. Fig. 5 shows the rat kidney medulla portion incubated with anti-WCH1/C which has been pre-incubated with a corresponding peptide immunogen, with a magnification factor of 100. Specificity of the antibody staining was confirmed by the disappearance of the staining (Fig. 5), by the pre-incubation with the peptide immunogen, which staining was observed in the apical domain of the cells of the collecting tubule (Fig. 4).

Fig. 6 shows the rat's medullar portion incubated with anti-WCH-1, with a high magnification factor of 400. Fig. 7 shows the rat's kidney cortical portion incubated with anti-WCH1/C, with a magnification factor of 100.

Immunofluorescence staining was observed only in the cortical and medullar collecting tubules but not observed in other nephron segments inclusive of proximal tubule, thin descending tubule or thin ascending limb (Fig. 4 and 7). Specificity of the antibody staining was confirmed by the lack of staining of the sections when antiserum was preincubated with the corresponding peptide immunogen (Fig. 5). Immunolocalization of WCH-1 along the nephron segments, together with RT-PCR localization of WCH-1 mRNA, indicate the exclusive expression of WCH-1 in the collecting tubule, contrary to CHIP 28, which expresses itself in the proximal tubule and the descending thin limb of Henle's loop (Denker, B. M., Smith, B. L., Kuhajda, F. P. & Agre, P. J. *Biol. Chem.* 263, 15634 - 15642 (1988)).

It is known that a minority of cells of the cortical collecting tubule are not stained, and that these cells are intercalated cells in which the water channels are not expressed (Handler, J. S. *Am. J. Physiol.* 255, F375 - F382 (1988)). According to the intracellular immunochemical localization examined at high magnification, the apical membrane of the cells of the collecting tubule were stained deeply, whereas basolateral sides of the cells were hardly stained (Fig. 6). As a result thereof, it was proved that WCH-1 was localized in the apical membrane of the cells of the collecting tubule. Interestingly, staining could be observed in the sub-apical region of the cell, in addition to intense staining in the apical membrane. Although spatial resolution is not high enough, this is indicative of the presence of the water channels in the sub-apical endosomal reservoir.

Proof that WCH-1 is a Water Channel

To determine the osmotic water permeability of oocytes injected with the WCH-1 transcript, volume increase caused by an imposed osmotic gradient was measured using videomicroscopy (Zhang, R. & Verkman, A. S. *Am. J. Physiol.* 260, C26 - C34 (1991)).

Capped cRNA was synthesized from WCH-1 in pSPORT vector using T7 RNA polymerase after linearization of the pSPORT vector. Oocytes were obtained from female *Xenopus laevis* and prepared as described in Dascal, N. *CRC Crit. Rev. Biochem.* 22, 317 - 373 (1987), then injected with 20 ng of water or 20 ng of WCH-1 RNA (1 $\mu\text{g}/\mu\text{l}$) and incubated at 18 °C. After 24 hours of incubation, the oocytes were transferred from 200 mOsm Barth's buffer to 70 mOsm Barth's buffer and osmotic volume increase was observed at 24 °C by videomicroscopy (Zhang, R. & Verkman, A. S. *Am. J. Physiol.* 260, C26 - C34 (1991)). The oocytes were viewed by light transmitted through an Olympus phase-contrast microscope and imaged on a Hamamatsu SIT camera connected to ARGUS-200 image processing system. Images were obtained and stored at 20 sec intervals.

Oocytes images were processed as described in Zhang, R. & Verkman, A. S. *Am. J. Physiol.* 260, C26 - C34 (1991) and the projection area of the oocytes was calculated by automatic summation. Relative volume (V/V_0) was calculated from the area at time 0 (A_0) and at time t (A) by :

$$V / V_0 = (A / A_0)^{3/2}$$

Osmotic water permeability (Pf) was determined from an initial slope of a time curve of V / V_0 ($d(V / V_0) / dt$), initial oocyte volume ($V_0 = 9 \times 10^{-4} \text{ cm}^3$), initial oocyte surface area ($S = 0.045 \text{ cm}^2$), and the molar volume of water ($V_w = 18 \text{ cm}^3/\text{mol}$) by :

$$\text{Pf} = [V_0 \times d(V / V_0) / dt] / [S \times V_w \times (\text{osm}_{\text{in}} - \text{osm}_{\text{out}})]$$

To examine the effects of mercurial and sulfhydryl reagents, oocytes were incubated in Barth's buffer containing 0.3 mM HgCl_2 for 5 minutes prior to Pf measurements. The recovery of the inhibition by reducing agents was examined by 15 minutes incubation in a Barth's buffer containing 5 mM β -mercaptoethanol following 5-minute incubation in HgCl_2 .

Fig. 8 shows an increase in osmotic water permeability of *Xenopus* oocytes, in which WCH-1 RNA has been injected. Fig. 8 shows time-dependent volume increase of oocytes injected with 20 ng WCH-1 RNA (WCH-1) and with water (Control). Fig. 8 b shows microphotographs of oocytes injected with WCH-1 RNA or with water (Control). Photos were taken in 20 sec intervals, shown in the order of left-to-right and top-to-bottom.

Osmotic water permeability (Pf) was 25.1 ± 1.7 (mean $\pm$ SEM) $\times 10^{-4} \text{ cm/s}$ in oocytes injected with water and $83.9 \pm 18.2 \times 10^{-4} \text{ cm/s}$ in oocytes injected with WCH-1. The osmotic water permeability

coefficient (Pf) in WCH-1-injected oocytes was 3.5 times greater than Pf in water-injected oocytes.

Moreover, ten out of eleven oocytes injected with WCH-1 transcripts ruptured within 10 minutes after transfer into the hypotonic solution, whereas none of water-injected oocytes ruptured for more than 60 minutes.

The Pf value in the oocytes injected with WCH-1, which was $83.9 \pm 18.2 \times 10^{-4}$ cm/sec, was lowered to $44.5 \pm 3.6 \times 10^{-4}$ cm/sec after incubation for five minutes in 0.3 mM of HgCl_2 , due to partial suppression of activity of the WCH-1 water channel. Suppression by HgCl_2 was recovered by incubation in 5 mM β -mercaptoethanol for 15 minutes following incubation in 0.3 mM of HgCl_2 (Pf = $63.1 \pm 25.8 \times 10^{-4}$ cm/sec).

The activation energy (Ea) for osmotic water permeability of oocytes injected with WCH-1 cRNA, estimated from the Arrhenius Plot of Pf measured at 10 to 28 °C was 3.6 ± 0.8 kcal/mol (n = 12), which is comparable to those reported for water channels in RBC and kidney proximal and collecting tubules (Verkman, A. S. *Annu. Rev. Physiol.* 54, 97 - 108 (1992)).

Appearance of high osmotic water permeability, and low activation energy, together with the inhibition by mercurial reagents acting on sulfhydryl groups and the recovery with reducing agents, which are characteristic to channel mediated water permeability (Verkman, A. S. *Annu. Rev. Physiol.* 54, 97 - 108 (1992)), strongly suggest that the expressed protein in the WCH-1-injected oocytes is a water channel.

Our observed Pf value in WCH-1-injected oocytes was lower than that reported for CHIP 28-injected oocytes. Possible explanations for it include reduced translation of WCH-1 protein without using a *Xenopus* β -globin chimaeric vector (Preston, G. M., Carroll, T. P., Guggino, W. B. & Agre, P. *Science* 256, 385 - 387 (1992)), and reduced surface expression of WCH-1 in oocytes due to the lack of the vasopressin-regulated membrane trafficking mechanisms, which are necessary for translocating water channels from subapical reservoir vesicles to the apical membrane (Handler, J. S. *Am. J. Physiol.* 255, F375 - F382 (1988)). Also, in *Xenopus* oocytes, endosomal water channel protein could only partially be expressed in the plasma membrane because of the non-specific targeting of foreign protein in the egg (Dascal, N. *CRC Crit. Rev. Biochem.* 22, 317 - 373 (1987); Sigel, E. J. *Membrane Biol.* 117, 201 - 221 (1990)).

To examine the effects of water deprivation, 50 μ g of RNA from rat whole kidney after 0, 2, and 5 day of water restriction were used. RNA was size fractionated by agaroseformaldehyde gel electrophoresis. Equal loading and absence of degradation were checked by staining with ethidium bromide and by hybridization with ^{32}P -labeled β -actin. After transfer to nylon membranes, blots were hybridized under high stringency with the WCH-1 cDNA for WCH-1 expression and the insert of prCHIP 28 for CHIP 28 expression labeled with ^{32}P , and autoradiographed.

Fig. 9 shows the results of Northern blot analysis of the regulation of WCH-1 and CHIP 28 mRNA abundance after prolonged water deprivation in Rat kidney.

Significant induction of WCH-1 mRNA, but not of CHIP 28 mRNA, in rat kidney after prolonged water deprivation suggests that during prolonged antidiuresis, an increment in collecting tubule water channel protein may contribute to the increase in water permeability.

The antidiuretic action of vasopressin includes rapid increases in water permeability of the collecting tubule by inserting water channels into the apical membrane (Ganote, C. E. et al. *J. Cell Biol.* 36, 355 - 367 (1968); Kuwahara, M., Berry, C. A. & Verkman, A. S. *Biophys. J.* 54, 595 - 602 (1988); Verkman, A. S. *Annu. Rev. Physiol.* 54, 97 - 108 (1992)), and an increased urinary concentration capacity by amplifying the countercurrent multiplication system and the corticomedullary osmolality gradient (Knepper, M. A. & Rector, F. C. Jr. in the *Kidney* (eds Brenner, B. M. & Rector, F. C. Jr.) 445 - 482 (W. B. Saunders, Philadelphia, 1991); Kirk, K. L. & Schafer, J. A. in the *Kidney: Physiology and Pathophysiology* (eds Seldin, D. W. & Giebisch, G.) 1693 - 1725 (Raven Press, New, York, 1992)).

Our results indicate the possibility that vasopressin may also increase maximal water permeability by increasing the synthesis of water channels in the collecting tubule.

Because vasopressin causes enormous increase in osmotic water permeability of the apical membrane of the collecting tubule from basal level, comparable to that of other biological membranes that do not contain water channels (Verkman, A. S. *Annu. Rev. Physiol.* 54, 97 - 108 (1992); Kuwahara, M., Berry, C. A. & Verkman, A. S. *Biophys. J.* 54, 595 - 602 (1988)), all or at least the majority of water channels in the apical membrane are considered to be vasopressin regulated. The presence of WCH-1 exclusively in the apical domain of the collecting tubule cells, and the functional expression of water channel in oocytes strongly indicate that WCH-1 is indeed the vasopressin-regulated water channel of the apical and endosomal membranes of the collecting tubule.

Molecular identification of the apical membrane water channel will enable direct investigation on the cellular mechanisms of the vasopressin-regulated water permeability of the collecting tubule cells.

Means for Obtaining WCH-1 Protein

Cyclic plasmid pSPORT is sectioned with restrictive enzymes Not-I and Sal-I, and WCH-1 cDNA is inserted into the sectioned sites. The plasmid into which WCH-1 gene has been recombined is introduced into an Escherichia coli DH10 α strain for transformation.

The transformation of host by the recombinant DNA may be realized by the known method of Cohen, S.N. et al., Proc. Natl. Acad. Sci., USA., 69, 2110 (1972) or a similar method.

The produced transformant or recombinant is cultivated on a known medium, such as (ampicillin-containing) L-broth medium. IPTG is added during cultivation in order for the promotor to be operated more effectively during a certain predetermined period following bacterial proliferation. After IPTG addition, cultivation is continued usually for 3 to 4 hours at 37 °C. After the cultivation, bacteria are collected by any known method, suspended in a buffer solution and ruptured. WCH-1 protein is purified by known methods, such as column chromatography.

Examples of Industrial Utilization

The following industrial advantages may be accrued by the isolation, identification and cloning of WCH-1 which are enabled by the present invention.

A screening method for water diuretics utilizing a substance in which the WCH-1 protein is expressed, such as following screening methods for water diuretics, may be established.

1) Several peptides thought to be the center of activity may be artificially synthesized from the amino acid sequence of WCH-1, and substances which are specifically linked to these peptides are screened.

2) A large quantity of WCH-1 protein is produced from

Escherichia coli having the WCH-1 recombinant plasmid according to the present invention and substances which are specifically linked to such protein are screened.

3) mRNA artificially produced from WCH-1 cDNA is injected into eggs of Xenopus to express WCH-1 protein on the egg membrane. Since the eggs are dilated on lowering of the osmotic pressure of the external liquid, the eggs are cultivated in a hypotonic liquid and the dilation is monitored to screen substances which are restrained in dilation.

4) An antibody against the extracellular domain of the WCH-1 protein is prepared, and substances which inhibit combination of the antibody with the WCH-1 protein are screened.

5) A vector in which WCH-1 cDNA is incorporated is transplanted on a suitable culture cell, such as COS-7 cell. Since the transplanted cell is improved in water permeability, water flows into the cell to be dilated and exploded on decreasing the osmotic pressure of the external liquid. Therefore, substances which restrict such explosion are screened.

An artificial membrane in which the WCH-1 protein is incorporated (Bear, C. E. et al., Cell, 68, 809 - 818 (1992)) exhibits high water permeability and may be used in a number of ways. For example,

1) The artificial membrane may be used as a membrane for ultrafiltration for preparation of pure water.

2) It may be used for salt concentration.

3) It may be used as an osmotic pressure sensor.

4) It may also be used for screening of water diuretics.

Above all, the WCH-1 protein incorporated into liposome as a type of the lipid membrane may also be used in a variety of ways. For example,

1) it may be designed as artificial blood cells:

2) it may be designed as the above-mentioned liposome in which pharmaceuticals are contained. Such liposome is suitable as a durable slow-release pharmaceutical because of retarded release of the pharmaceuticals in the blood;

3) it may be used as a therapeutic drug for patients showing resistivity to water diuretics and for patients suffering from severe edema.

If liposome containing high osmotic pressure substances is ingested, body water is captured into liposome and ultimately excreted as feces, in other words, water discharge into feces is promoted.

4) The liposome may be used as a water absorbant.

The liposome containing high osmotic pressure substances exhibit high water absorption properties. Therefore, if a large quantity of the liposome are filled between paper cells of a paper product, such paper product is markedly improved in water adsorptive properties, and hence may be used as a diaper or a sanitary napkin.

The liposome into which WCH-1 protein is incorporated may be produced by any of known methods. Among the common methods, there are a freezing melting method (M. Kasahara, P. C. Hinkle, J. Biol.

Chem., 252, 7384 (1977)), a dilution method by octylglucoside (M. J. Newman, T. H. Wilson, J. Biol. Chem., 255, 10583 (1980)) and a dialysis method (Y. Kagawa, A. Kandrach, E. Racker, J. Biol. Chem., 248, 676 (1973)).

The size and properties (i.e. single- or multi-layered) of the liposome are suitably selected depending on lipid types.

As an example, the WCH-1 protein, the lipid (preferably as a mixture with phospholipid) and a surfactant (e.g. deoxycholate) are mixed together and agitated ultrasonically. The surfactant is removed by dialysis or gel filtration to produce liposome into which is incorporated the WCH-1 protein.

An example of liposome preparation by the freezing and melting method is explained.

Preparation Example

To 100 mM of tris-hydrochloric acid buffer solution (pH 7.5), 50 mM of $MgCl_2$ and 22.5 mg of azolectin (crude lipid of soybeans) processed with acetone are added 0.5 ml of a 10 mM tris-hydrochloric acid buffer solution (pH 7.5). After blowing nitrogen gas into the resulting mixture, the mixture is treated for about 20 minutes by a water-bath type ultrasonic vibrator by ultrasonic waves until the mixture is substantially transparent.

167 μ l of this liposome, 20 μ g of purified WCH-1 protein and 10 mM of tris-hydrochloric acid buffer solution (pH 7.5) are combined to an overall volume of 0.5 ml. After blowing nitrogen gas into the resulting mass, the mass is frozen in acetone cooled to $-70^\circ C$. After melting at room temperature, the melted mass is ultrasonically treated for 15 sec using a water-bath type ultrasonic vibrator. The so-treated mass is diluted with 50 mM $MgCl_2$ - 100 mM tris-hydrochloric acid buffer solution (pH 7.5) and water to produce liposomes in which WCH-1 protein is incorporated in the form of 8 mg lipid in 1 ml of 2 mM $MgCl_2$ - 10 mM tris-hydrochloric acid buffer solution.

On the other hand, since it now becomes possible to duplicate the WCH-1 cDNA according to the present invention and to synthesize WCH-1 mRNA artificially, it becomes possible to express the WCH-1 protein on a living membrane by employing a known technique as disclosed in M. Mishina et al., Nature (London), 307, 604 (1984).

For example, the WCH-1 protein may be expressed on the cell membrane by transplanting the plasmid in which WCH-1 cDNA is incorporated on the cell medium or by injecting WCH-1 mRNA into oocytes, as shown in the above exemplification.

Appendix:

Table of Sequence Description

Table of Sequence Description

Sequence ID No. : 1

Sequence Length : 1408

Sequence Type : nucleic acid

Strandedness : single

Topology : linear

Molecule Type : cDNA to mRNA

Original Source

Organism : Sprague-Dawley rat

Individual Isolate of Clone : WCH-1

Tissue Type : Kidney

Immediate Source

Clone : pMWC41

Sequence Description

AGAGAGAAGA GAAAGAGAGA GGGAGGGAGG AAGAGCCACC CCGTGGCCC AGACCCCTGG 60

CCAGCGCGCA GAAGTCGGAG CAGC ATG TGG GAA CTC AGA TCC ATA GCC TTC 111

Met Trp Glu Leu Arg Ser Ile Ala Phe

1

5

TCC CGA GCA GTG CTG GCT GAG TTC TTG GCC ACG CTC CTT TTT GTC TTC 159

Ser Arg Ala Val Leu Ala Glu Phe Leu Ala Thr Leu Leu Phe Val Phe

10

15

20

25

TTT GGC CTT GGC TCA GCC CTC CAG TGG GCC AGC TCC CCA CCC TCT GTG 207

Phe Gly Leu Gly Ser Ala Leu Gln Trp Ala Ser Ser Pro Pro Ser Val

30

35

40

CTC CAG ATC GCC GTG GCC TTT GGT CTG GGC ATC GGC ATC CTG GTT CAG 255

Leu Gln Ile Ala Val Ala Phe Gly Leu Gly Ile Gly Ile Leu Val Gln

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EP 0 591 789 A2

	GCT CTG GGC CAT GTC AGC GGG GCA CAC ATC AAC CCC GCC GTG ACT GTG	303
5	Ala Leu Gly His Val Ser Gly Ala His Ile Asn Pro Ala Val Thr Val	
	60 65 70	
	GCA TGC CTG GTG GGT TGC CAT GTC TCC TTC CTT CGA GCT GCC TTC TAT	351
10	Ala Cys Leu Val Gly Cys His Val Ser Phe Leu Arg Ala Ala Phe Tyr	
	75 80 85	
	GTG GCT GCC CAG CTG CTG GGC GCC GTG GCT GGG GCT GCC ATC CTC CAT	399
15	Val Ala Ala Gln Leu Leu Gly Ala Val Ala Gly Ala Ala Ile Leu His	
	90 95 100 105	
	GAG ATT ACT CCA GTA GAA ATC CGT GGG GAC CTG GCT GTC AAT GCT CTC	447
20	Glu Ile Thr Pro Val Glu Ile Arg Gly Asp Leu Ala Val Asn Ala Leu	
	110 115 120	
	CAC AAC AAC GCC ACA GCT GGC CAG GCT GTG ACT GTA GAG CTC TTC CTG	495
25	His Asn Asn Ala Thr Ala Gly Gln Ala Val Thr Val Glu Leu Phe Leu	
	125 130 135	
	ACC ATG CAG CTG GTG CTG TGC ATC TTT GCC TCC ACC GAC GAG CGC CGC	543
30	Thr Met Gln Leu Val Leu Cys Ile Phe Ala Ser Thr Asp Glu Arg Arg	
	140 145 150	
	GGT GAC AAC CTG GGT AGC CCT GCC CTC TCC ATT GGT TTC TCT GTT ACC	591
35	Gly Asp Asn Leu Gly Ser Pro Ala Leu Ser Ile Gly Phe Ser Val Thr	
	155 160 165	
	CTG GGC CAC CTC CTT GGG ATC TAT TTC ACC GGT TGC TCC ATG AAT CCA	639
40	Leu Gly His Leu Leu Gly Ile Tyr Phe Thr Gly Cys Ser Met Asn Pro	
	170 175 180 185	
	GCC CGC TCC CTG GCT CCA GCA GTT GTC ACT GGC AAG TTT GAT GAT CAC	687
45	Ala Arg Ser Leu Ala Pro Ala Val Val Thr Gly Lys Phe Asp Asp His	
	190 195 200	
50		
55		

TGG GTC TTC TGG ATC GGA CCC CTG GTG GGC GCC ATC ATC GGC TCC CTC 735
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 205 210 215
 CTC TAC AAC TAC CTG CTG TTC CCC TCG GCA AAG AGC CTG CAG GAG CGC 783
 Leu Tyr Asn Tyr Leu Leu Phe Pro Ser Ala Lys Ser Leu Gln Glu Arg
 220 225 230
 TTG GCA GTG CTC AAG GGC CTG GAG CCC GAC ACC GAC TGG GAG GAA CGT 831
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 235 240 245
 GAA GTG CGG CGG CGG CAG TCG GTG GAG CTC CAC TCT CCT CAG AGC CTG 879
 Glu Val Arg Arg Arg Gln Ser Val Glu Leu His Ser Pro Gln Ser Leu
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 CCT CGC GGC AGC AAG GCC TGAGCTCCCC TGCAGCGCAC CGCAGCTCAG 927
 Pro Arg Gly Ser Lys Ala
 270 271
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 GCTTTGGCCT TCTTCTCCTG TACCTGCAAT AAATCCACTA TCTCTGAAAA AAAAAAAAAA 1407
 A 1408

Claims

1. A WCH-1 cDNA probe specific to mRNA expressing a water channel localized in the kidney collecting tubule and complementary to said mRNA.

2. A WCH-1 cDNA probe specific to mRNA expressing a vasopressin-regulated water channel and complementary to said mRNA.
3. The sequence of WCH-1 cDNA as defined in claim 1 wherein the sequence is expressed by a base sequence shown by sequence number 1.
4. A WCH-1 mRNA probe obtainable by employing the WCH-1 cDNA probe as defined in one of claims 1 to 3 as a template.
5. The WCH-1 cDNA as defined in one of claims 1 to 3 obtainable by
 - a) subjecting a single-chain cDNA prepared from kidney medullary mRNA of a mammal to PCR using, as degenerate primers,

5' - (T/C)T(T/C/A/G)AA(T/C)CC(T/C/A/G)GC(T/C/A/G)GT
(T/C/A/G)AC - 3'

and

5' - AA(T/C/A/G)(G/C)(T/A)(T/C/A/G)C(G/T)(T/C/A/G)GC(T/C/A/G)
GG(A/G)TT - 3',

- and
 - b) screening a kidney cDNA library of said mammal using a product of said PCR as a probe.
6. The WCH-1 cDNA as defined in claim 5 wherein the mammal of claim 5 is a rat.
7. A WCH-1 protein molecule constituting a water channel localized in the kidney collecting tubule.
8. A WCH-1 protein molecule constituting a vasopressin-regulated water channel.
9. An amino acid sequence of the WCH-1 protein molecule as defined in claims 7 or 8, wherein the amino acid sequence is coded by a base sequence of sequence number 1.
10. A recombinant plasmid characterized in that a WCH-1 gene represented by a base sequence shown by sequence number 1 is recombined into an expressing vector.
11. The recombinant plasmid as defined in claim 10 characterized in that said expressing vector is pSPORT, and said WCH-1 gene is inserted into sites of said pSPORT sectioned with Not-I and Sal-I.
12. Escherichia coli producing protein molecules expressed by the WCH-1 cDNA as defined in claim 6, said protein molecules constituting a water channel localized in the kidney collecting tubule.
13. Escherichia coli producing protein molecules expressed by the WCH-1 cDNA as defined in claim 6, said protein molecules constituting a vasopressin-regulated water channel.
14. Escherichia coli producing protein molecules expressed by WCH-1 gene represented by a base sequence shown by sequence number 1, said protein molecules constituting a water channel localized in the kidney collecting tubule.
15. Escherichia coli producing protein molecules expressed by WCH-1 gene represented by a base sequence shown by sequence number 1, said protein molecules constituting a vasopressin-regulated water channel.

16. The *Escherichia coli* as defined in any one of claims 12 to 15 obtainable by introducing the recombinant plasmid obtained by inserting the WCH-1 gene into the expressing vector pSPORT into an *Escherichia coli* DH10 α for transformation.

5 17. The *Escherichia coli* as defined in claim 16 containing the plasmid in which said WCH-1 gene is introduced into sites of pSPORT sectioned with Not-I and Sal-I.

10 18. The *Escherichia coli* having the plasmid as defined in claim 17, produced by employing the vector (pSPORT), the expression of which may be derived by addition of isopropyl β -D-thiogalactoside, host (DH10 α) family.

19. A WCH-1 protein molecule produced by *Escherichia coli* as defined in any one of claims 1 to 18.

15 20. A method for producing WCH-1 protein molecules characterized in that the WCH-1 protein molecules are obtained using *Escherichia coli* as defined in any one of claims 12 to 18.

21. A lipid membrane characterized in that it contains WCH-1 protein molecules constituting a water channel localized in the kidney medullary tubule.

20 22. A lipid membrane characterized in that it contains WCH-1 protein molecules constituting a vasopressin-regulated water channel.

23. A liposome characterized in that it is formed of a lipid membrane containing WCH-1 protein molecules constituting a water channel localized in the kidney medullary tubule.

25 24. A liposome characterized in that it is formed of a lipid membrane containing WCH-1 protein molecules constituting a vasopressin-regulated water channel.

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Fig. 1

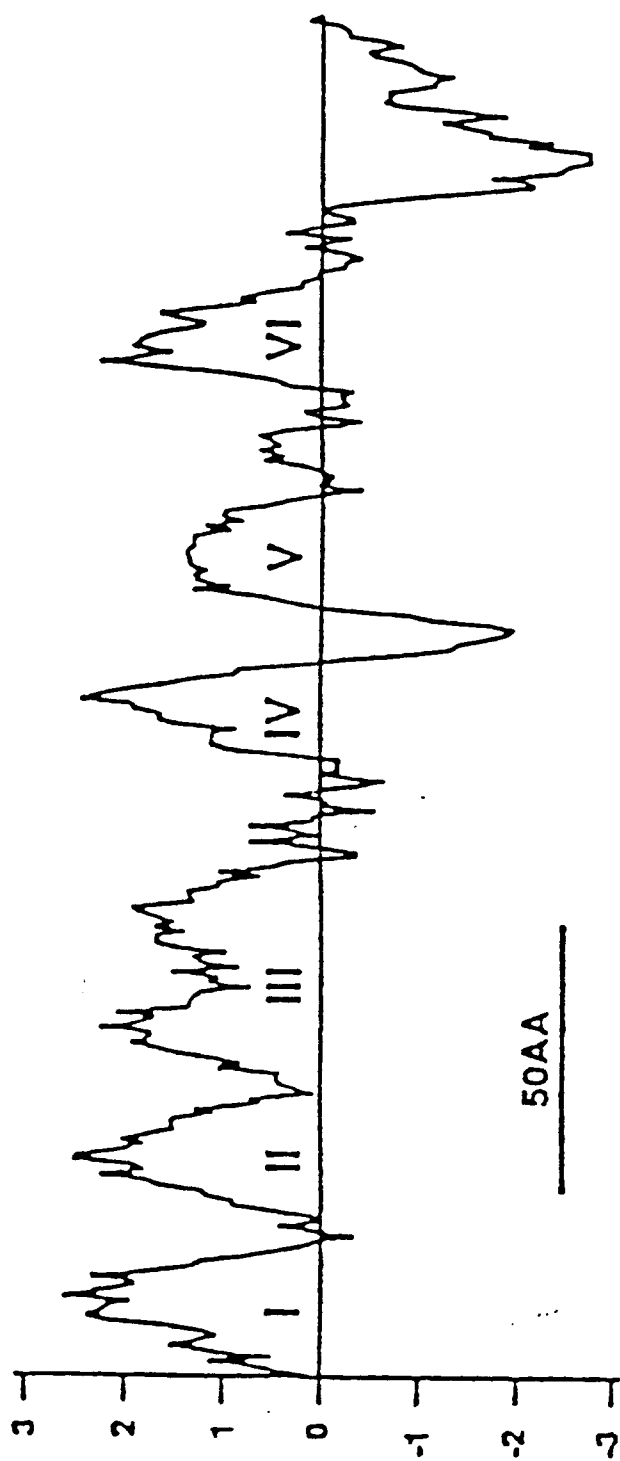
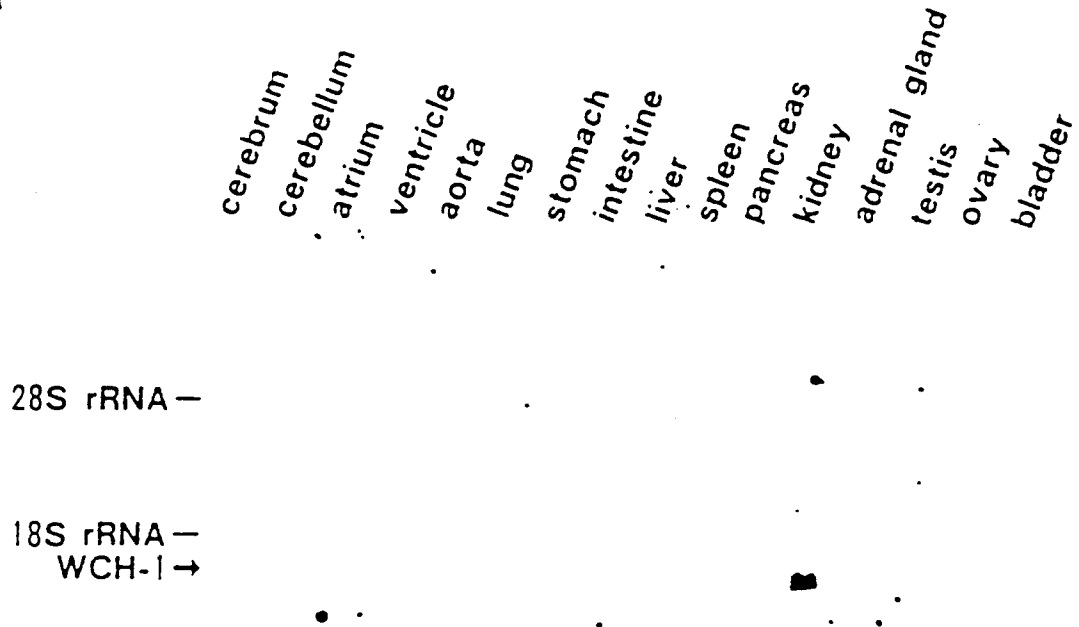


Fig. 2

(a)

a



(b)

b

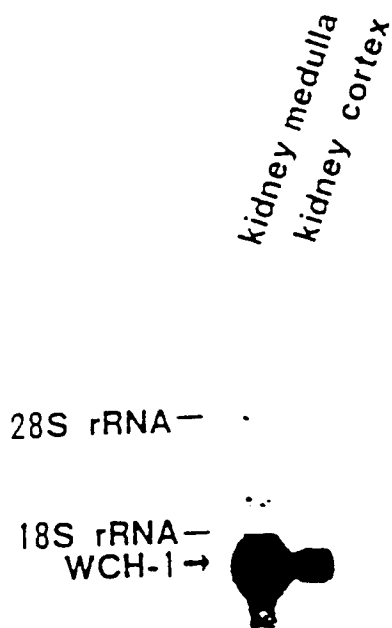
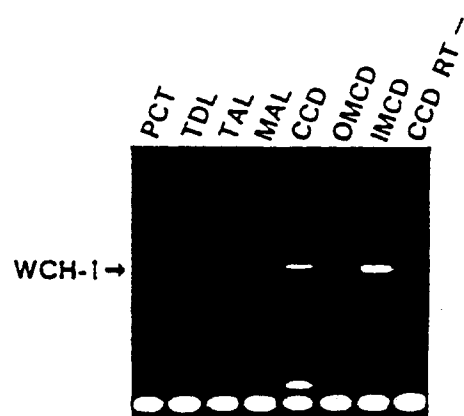
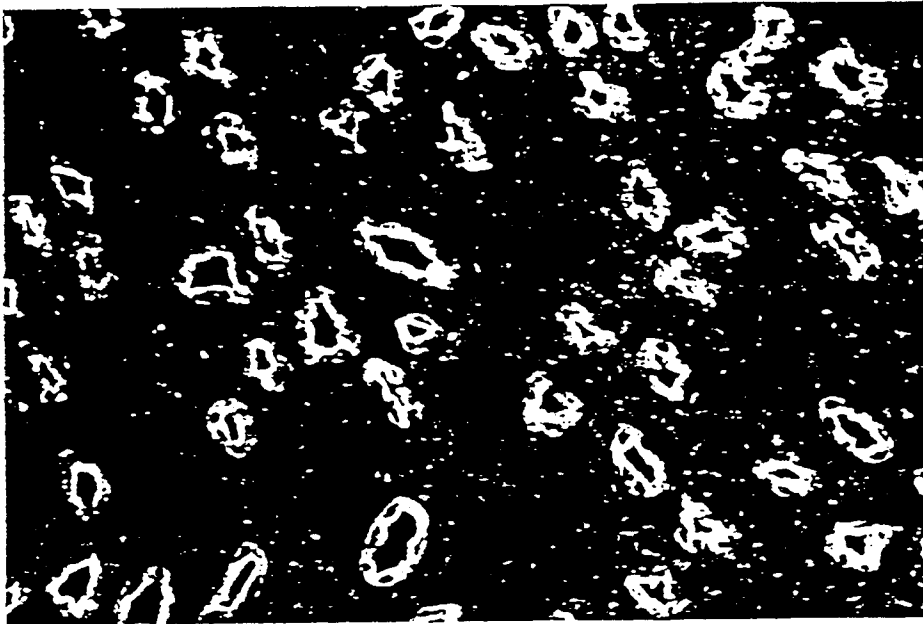


Fig. 3

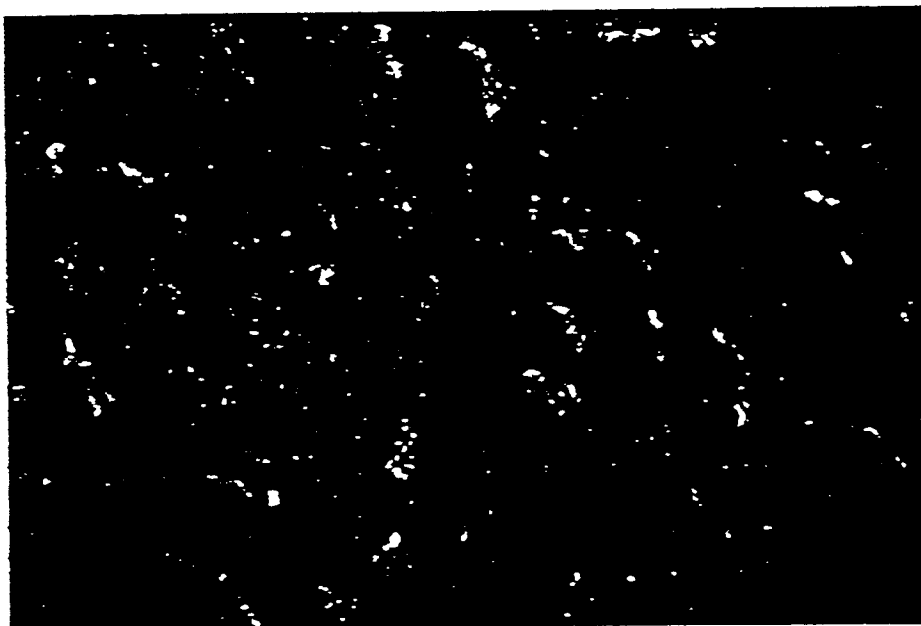
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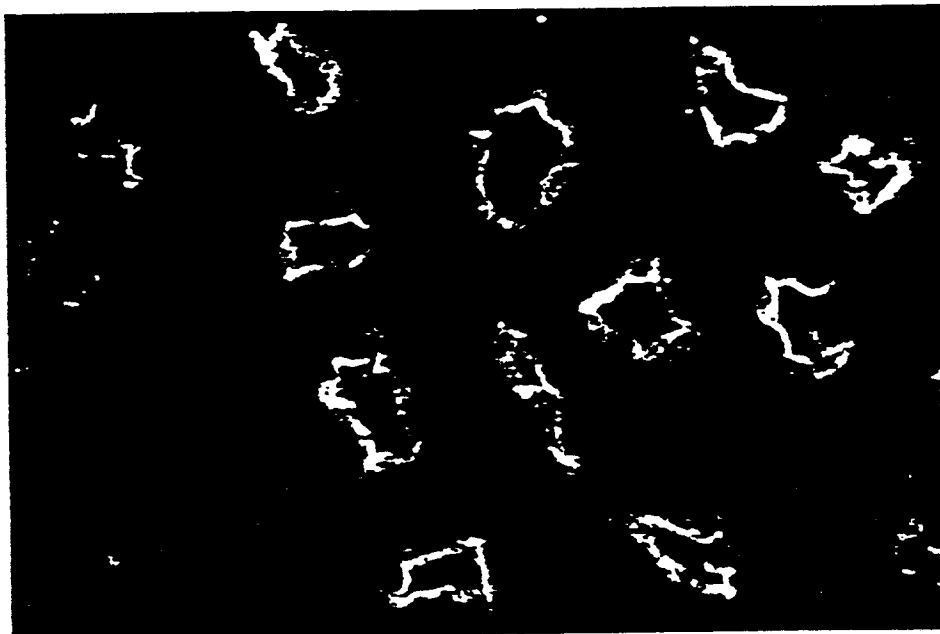
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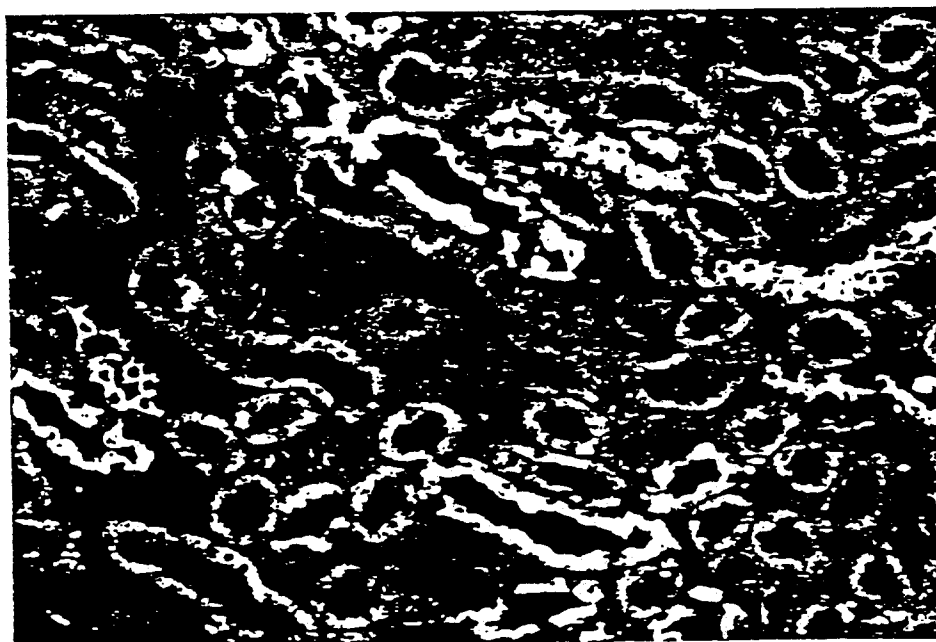
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F i g . 6

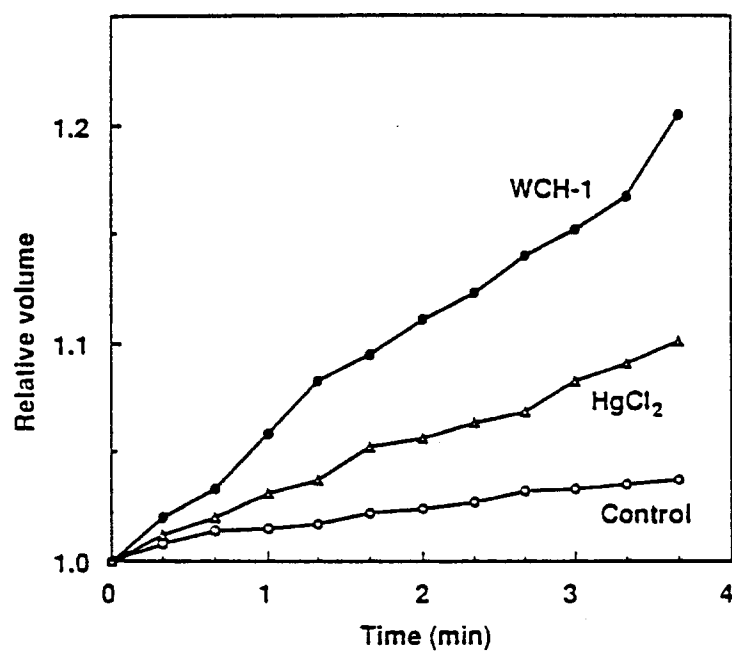


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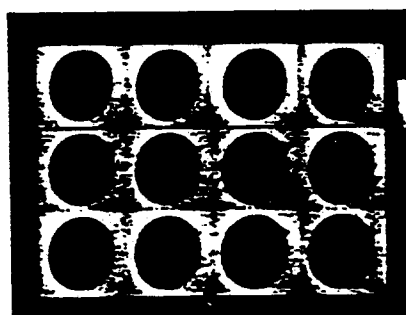


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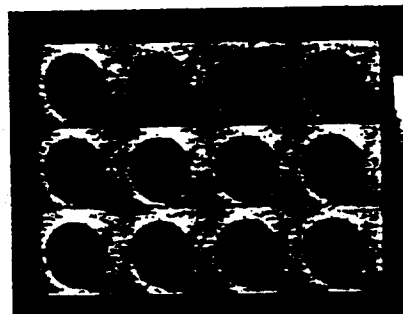
(a)



(b)

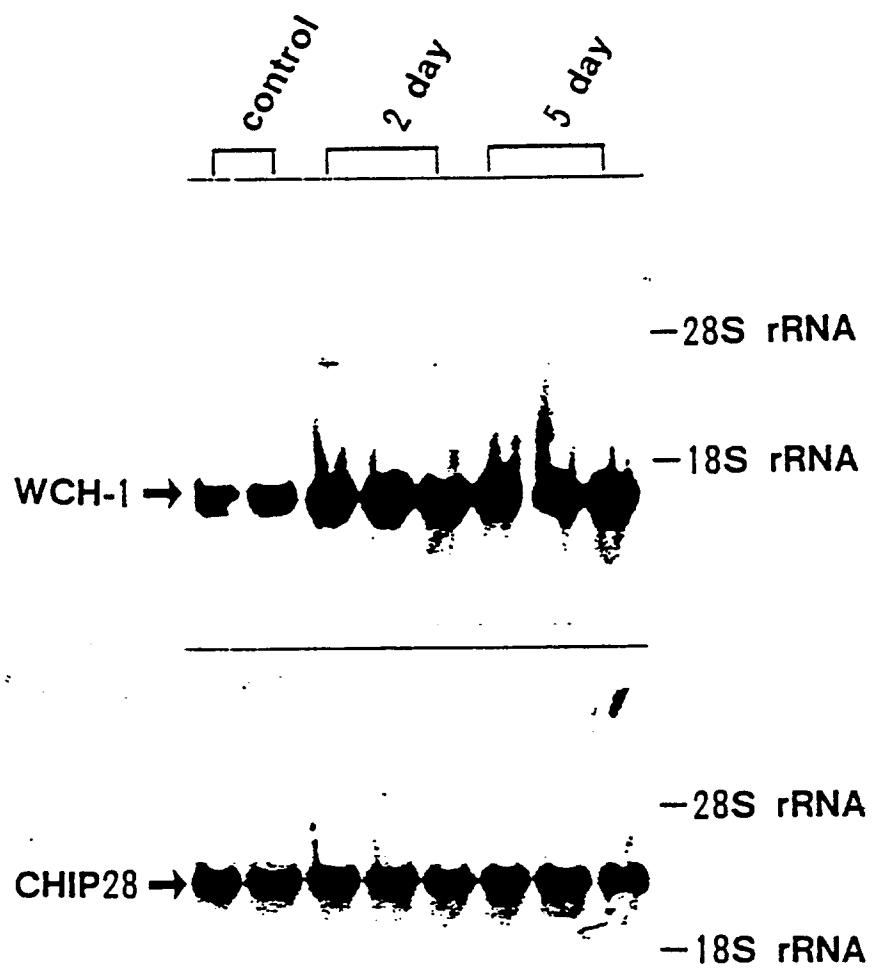


WCH-1



Control

F i g . 9



F i g . 1 0

		-84 AGACAGACAGACAGACAGAC	-64
		GGAGGAGCAAGAGCCACCCCTGGCCGAGACCCCTGGCCAGCCGAGCAAGTGGAGC	-4
WCH-1		AGCATGTGGAACTCAGATCCATAGCTTCTCGGAGCAGTGGTGGTGAATCTTGGCC	57
CHIP28		M W E L R S I A F S R A V L A E F L A	19
		M A S E F K K K L F W R A V V A E F L A	20
WCH-1		ACGCTGCTTCTGTCTTCTTGGCCCTGGGTCAGCCCTC	96
CHIP28		T L L F V F F G L G S A L - - - - -	32
		T T L F V F I S I G S A L C F K Y P V G	40
WCH-1		CAGTGGGTCAGTCCGACCTGTGTGCTCCAGATCCGCTGGCTTGGTCTGGGCATC	156
CHIP28		Q W A S S P P S V L Q I A V A F G L G I	52
		N N Q T A V Q D N V K V S L A F G L S I	60
WCH-1		GGCATGCTGCTTCAAGCTGTCTGGCCATGTCCAGCGGGACACATCAAGCGCGCTGACT	216
CHIP28		G I L V Q A L G H V S G A H I N P A V T	72
		A T L A Q S V G H I S G A H L N P A V T	80
WCH-1		GTGGCATGCTGCTGGCTTGGCATGTCTGCTTGGTCCAGATGCTTCTATGTGGTGGC	276
CHIP28		V A C L V G C H V S F L R A A F Y V A A	92
		L G L L S C Q I S I F R A L M Y I I A	100
WCH-1		CAGTGTGGGCGCTGGCTGGGCTGGCCATGCTCCATGAGATTACTCCAGTAGAAATC	336
CHIP28		Q L L G A V A G A A I L H E I T P V E I	112
		Q C V G A I V A T A I L S G I T S S L T	120
WCH-1		CGTGGGACCTGGCTGTCAATGCTCTCCACAACAGCCGACAGCTGGCCAGGCTGTGACT	396
CHIP28		R G D L A V N A L H N A T A G Q A V T	132
		G N S L G R N D L A D G V N S G Q G L G	140
WCH-1		GTAGACCTCTCTGACCATGCAAGTGGTGTGGATCTTGGCTCCACCGACGAGGGC	456
CHIP28		V E L F L T M Q L V L C I F A S T D E R	152
		I E I I G T L Q L V L C V L A T T D R R	160
WCH-1		CGGGGTGACAGCTGGGTAGCCCTGGCTCTGCAATGGTCTGTCTTACGCTGGGCGAC	516
CHIP28		R G D N L G S P A L S I G F S V T L G H	172
		R R D L G G S A P L A I G L S V A L G H	180
WCH-1		GTCTTGGGATCTATTTACGCGGTGGTCCATGAATCCAGCGCGCTCCCTGGCTCCAGCA	576
CHIP28		L L G I Y F T G C S M N P A R S L A P A	192
		L L A I D Y T G C G I N P A R S F G S A	200
WCH-1		GTCTGACTGGCAAGTTGATGATCACTGGTGTCTGGATCGSACCGCTGGTGGGCGCC	636
CHIP28		V V T G K F D D H W V F W I G P L V G A	212
		V I T H N F S N H W I F W V G P F I G G	220
WCH-1		ATCATGGGCTCCCTCTCTCAACTACCTGGTGTCTGGCTGGGCAAGAGCTGCAGGAG	696
CHIP28		I I G S L L Y N Y L L F P A K S L Q E	232
		A L A V L I Y D F I L A P R S S D L T D	240
WCH-1		CGGTTGGGAGTGTCAAGCGCGTSSAGCCGACCCGACTGGGAGGAACGTGAAGTG	753
CHIP28		R L A V L K G L E P D T - D W E E R E V	251
		R V N V W T S G Q V E E Y D L D A D D I	260
WCH-1		CGGCGGCGGAGCTGGGTGGAGTCCACTCTCTTCAGAGCTGCTCTGGGAGCAAGGCC	813
CHIP28		R R R Q S V E L H S P Q S L P R G S K A	271
		N S R V E M K P K	269
		TGAGTCCGCTGCAAGGACCCGAGTCAAGCCGACGAGCTGGGCGCTGGTGGCTGGTGGC	873
		TGAGCGGTGGTGGTTCCAGTCCAGAGTAGCTGCTCCAGCGAGTGCAGTGAAGTCAAG	933
		AAGGGGTGGGCGGAGTGCAGTACCTTCCGCGGGAAGCGTTGAGTACCTTCAGGCT	993
		CGGCGTTGAGGAACGACAGCTTGGGCAAGGAGCTGGGCAAGGAGGAGCGCGCT	1053
		GACAGCGGAGAGCTTGGAGAGCCGCTTGGTGGTGGGAGAACTGCATAGACTGCT	1113
		TCTGGGAGCTGTGCTTAGTGCATCTCATTTATTAGTTGTAAAAGTGGTGGTCTGGC	1173
		GTATTTCTTTCTCAGCAACAGAGTTGCATGATCTGAGCTGATCCGAGTGGTGT	1233
		GGTGATACAGAGCGGGAGCTGCTATTCGCGTTGGGCTTCTTCTCTGTACCTGCAAT	1293
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